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Effect of PANTA on Growth of *Mycobacterium kansasii* in BACTEC 12B Medium

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***Mycobacterium kansasii* isolates from two patients showed relatively slow growth in BACTEC 12B medium (12B) (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) compared with the more rapid growth of these isolates on solid media. This finding prompted an evaluation of the effect of PANTA (Becton Dickinson) on the growth rate of these isolates. Suspensions of one isolate from each of these two patients (A and B), six additional isolates from six other patients (C through H), and one *M. kansasii* American Type Culture Collection isolate were inoculated into 12B with PANTA, 12B with reconstituting fluid only, and Middlebrook 7H11 agar plates (Remel, Lenexa, Kans.). For the isolates from patients A and B, the average times to detection for 12B with PANTA, 12B with reconstituting fluid, and Middlebrook 7H11 agar were 12.3, 7.4, and 9.0 days, respectively. For the remaining six patient isolates and the American Type Culture Collection strain, the average times to detection for these media were 9.2, 8.1, and 9.6 days. Susceptibility tests performed with the isolates from patients A and B with the individual component antibiotics of PANTA and testing of four of the other isolates with nalidixic acid alone suggested that nalidixic acid exerts some degree of inhibition on the growth of *M. kansasii*. The eight patient isolates were also inoculated onto Lowenstein Jensen medium (Remel) and onto a variety of selective mycobacterial media containing nalidixic acid and other antimicrobial agents. All isolates showed some degree of inhibition on at least one of these selective media.**

BACTEC 12B medium (12B) is widely used as a primary isolation medium for mycobacteria. Specimens from sterile body sources may be inoculated directly into 12B; specimens which contain contaminating flora are digested and decontaminated before inoculation into the bottles. 12B bottles inoculated with such processed specimens also receive a 0.1-ml aliquot of PANTA (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.), a mixture of antibiotics and reconstituting fluid which is intended to suppress the growth of contaminating organisms which may have survived the decontamination process (1a, 5). The components of PANTA and their final concentrations in 12B are as follows: polymyxin B, 50 U/ml; amphotericin B, 5 μg/ml; nalidixic acid, 20 μg/ml; trimethoprim, 5 μg/ml; and azlocillin, 10 μg/ml.

In our laboratory, patient specimens from contaminated sites are inoculated into 12B with PANTA and onto Middlebrook agar plates (Remel, Lenexa, Kans.) after processing. In most cases, growth is detected first from 12B. Recently, growth of *Mycobacterium kansasii* was detected first on Middlebrook plates from specimens from two patients. This prompted an investigation into the possible presence of an inhibitory factor for *M. kansasii* in PANTA or in 12B itself.

(A portion of these data was presented previously [1].)

MATERIALS AND METHODS

Organisms. Eight *M. kansasii* isolates from eight patients (A through H) at the Warren G. Magnuson Clinical Center of the National Institutes of Health and one control organism, *M. kansasii* ATCC 12478, were used in this study. All patient isolates except the isolate from patient C were identified by DNA hybridization (Gen Probe, San Diego, Calif.). The isolate from patient C was probe negative and was biochemically typical of *M. kansasii*, except for the 10-day Tween hydrolysis test, which was negative. The identification was subsequently confirmed biochemically by the National Jewish Center for Immunology and Respiratory Medicine (Denver, Colo.).

Evaluation of growth in BACTEC bottles and on Middlebrook agar. Suspensions of each *M. kansasii* isolate approximating a 0.5 McFarland standard were prepared and diluted to 10^4-fold in 7H9 broth (Difco, Detroit, Mich.).

Each isolate was tested with three lots of PANTA and three lots of reconstituting fluid. Six 12B bottles were prepared for each isolate, with three bottles receiving 0.1 ml of the different lots of PANTA and the other three receiving 0.1 ml of the different lots of reconstituting fluid. The 12B bottles and one Middlebrook 7H11 agar plate were each inoculated with 0.1 ml of the 10^4-fold suspension dilution. The Middlebrook plates were incubated at 35°C in 6% CO_2 and examined macroscopically for growth every other day, beginning on day 3, until growth was detected. Bottles were incubated at 35°C and tested with the BACTEC 460 instrument (Becton Dickinson Diagnostic Instrument Systems) every other day, beginning on day 0, until a growth index (GI) of 20 was achieved, at which time the bottles were subcultured to Middlebrook and horse blood agar (Remel) to check for contaminating organisms.

Susceptibility testing. (i) Antibiotics. The individual antibiotic components of PANTA were added to 12B bottles to achieve final concentrations equal to those obtained when PANTA is used. Additional twofold antibiotic dilutions, one above and one below these initial concentrations, were also tested. The antibiotics and final concentrations used were as follows: polymyxin B (Sigma, St. Louis, Mo.), 25, 50, and 100 U/ml; amphotericin B (Sigma), 2.5, 5, and 10 μg/ml; nalidixic acid (Sigma), 10, 20, and 40 μg/ml; trimethoprim (Sigma), 2.5, 5, and 10 μg/ml; and azlocillin (Sigma), 5, 10, and 20 μg/ml. Antibiotic dilutions were prepared in 7H9 broth. All concentrations of all antibiotics were tested against the isolates from patients A and B. Isolates from patients C, D, E, and G were arbitrarily selected for susceptibility testing with nalidixic acid only.

(ii) Organism suspensions. A suspension approximating a 0.5 McFarland standard was prepared in 7H9 broth for each organism to be tested. A 1:20 dilution of each of these suspensions was prepared in 7H9 broth, and 0.1-ml aliquots were inoculated into the drug-containing bottles and into a drug-free control bottle.

To provide the type of drug-free growth conditions suggested by the manufacturer for interpreting susceptibility tests with *M. kansasii*, 0.1 ml of the 1:20 dilution was also added to 9.9 ml of 7H9 broth to prepare a further 1:100 dilution. A portion (0.1 ml) of this dilution was inoculated into a drug-free 12B bottle. All bottles were incubated at 35°C and were tested daily on the BACTEC 460 instrument until the bottle containing the 1:100 dilution attained a GI of at least 30.

Analysis of susceptibility data. Interpretations of susceptibility or resistance were made according to the criteria of the manufacturer.

In addition, the difference in GI values between the day 2 and day 3 readings (delta value) was calculated for each drug-containing and drug-free control 12B bottle (with day 0 being the day of inoculation). This testing interval was chosen

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TABLE 1. Average number and range of days to detection of *M. kansasii* isolates with three lots of PANTA, reconstituting fluid only, and on Middlebrook 7H11 agar

Patient or other isolate	Avg (range) no. of days to detection on:		
	PANTA	Reconstituting fluid	Middlebrook 7H11 agar
A	12.3 (11–13)	7.0 (7)	9.0
B	12.3 (11–13)	7.7 (7–9)	9.0
Avg of A and B	12.3	7.4	9.0
C	9.0 (9)	7.0 (7)	9.0
D	7.0 (7)	7.0 (7)	9.0
E	9.0 (7–11)	7.0 (7)	9.0
F	13.0 (13)	13.0 (13)	11.0
G	7.0 (7)	7.0 (7)	9.0
H	7.0 (7)	7.0 (7)	9.0
ATCC 12478	12.3 (11–13)	9.0 (9)	11.0
Avg of C to H and ATCC 12478	9.2	8.1	9.6

because it was the one interval which could be used for all concentrations of all isolates. The delta values obtained for the isolates from patients A and B tested with a specific antibiotic dilution were averaged and expressed as a percentage of the average of the delta values of the drug-free controls for the same isolates. The isolates from patients C, D, E, and G were handled similarly with regard to their nalidixic acid susceptibility results.

Evaluation of growth on selective media. Suspensions approximating a 0.5 McFarland standard were prepared for each of the eight patient isolates and diluted to 10^3-fold in 7H9 broth. A portion (0.3 ml) of each 10^3-fold suspension was used to inoculate one tube of each of the following media: Lowenstein Jensen (LJ [Remel]), LJ Gruft (nalidixic acid, 35 μg/ml; penicillin, 50 U/ml [Remel]), Mycobactosel (nalidixic acid, 20 μg/ml; cycloheximide, 360 μg/ml; lincomycin, 2 μg/ml [Becton Dickinson Microbiology Systems, Cockeysville, Md.]), and Mycobactosel LJ (nalidixic acid, 35 μg/ml; cycloheximide, 400 μg/ml; lincomycin, 2 μg/ml [Becton Dickinson]). Tubes were incubated at 35°C in 8% CO_2 and examined for growth on days 11, 14, 18, 27, and 36.

RESULTS

Evaluation of growth in BACTEC bottles and on Middlebrook agar. The isolates from patients A and B showed a similar degree of inhibition by PANTA. For these isolates, the average time to detection from 12B with PANTA was 12.3 days (range, 11 to 13 days). The average times to detection for these isolates from 12B with reconstituting fluid only and from Middlebrook agar were 7.4 days (range, 7 to 9 days) and 9.0 days (range, 9 days), respectively. For the isolates from patients C through H and the American Type Culture Collection isolate, the average times to detection from the various media were as follows: 12B with PANTA, 9.2 days (range, 7 to 13 days); 12B with reconstituting fluid, 8.1 days (range, 7 to 13 days); Middlebrook agar, 9.6 days (range, 9 to 11 days) (Table 1).

Horse blood and Middlebrook 7H11 subcultures of 12B bottles showed all bottles to be free from contaminating organisms.

Susceptibility testing. With the manufacturer's guidelines for the interpretation of radiometric susceptibility tests, the isolate from patient A was determined to be susceptible to nalidixic acid at a concentration of 40 μg/ml and resistant to all concentrations of all other drugs tested. With the same criteria, all other isolates tested were calculated to be resistant to all concentrations of all of the drugs tested.

However, for all isolates, the delta values for nalidixic acid were a much smaller percentage of the corresponding delta values for the drug-free controls than were the delta values and percentages of control delta values for the other drugs tested.

TABLE 2. Average delta value of selected isolates with all concentrations of drugs

Drug	Concn[a]	Avg (avg % of control) delta value for patients:	
		A and B	C, D, E, and G
Polymyxin B	25	304 (105)	NT[b]
	50	290 (100)	NT
	100	251 (87)	NT
	Control	288	NT
Amphotericin B	2.5	270 (95)	NT
	5	286 (101)	NT
	10	284 (100)	NT
	Control	284	NT
Nalidixic acid	10	74 (27)	241 (92)
	20	48 (18)	159 (61)
	40	16 (6)	61 (23)
	Control	272	261
Trimethoprim	2.5	313 (101)	NT
	5	292 (94)	NT
	10	238 (77)	NT
	Control	310	NT
Azlocillin	5	472 (108)	NT
	10	452 (103)	NT
	20	446 (102)	NT
	Control	438	NT

[a] Units per milliliter for polymyxin B and micrograms per milliliter for all other drugs.
[b] NT, not tested.

These results suggested some inhibition by nalidixic acid of all of the *M. kansasii* isolates tested. To compare the results obtained with the various drug concentrations, the average delta values and percentage of control for the isolates tested at a particular drug concentration were calculated (Table 2). Average delta values for all 12B bottles containing the various concentrations of polymyxin B, amphotericin B, trimethoprim, and azlocillin ranged from 77 to 108% of the average delta value for the drug-free control bottles. For nalidixic acid, the average delta values obtained with the isolates from patients A and B ranged from 27% of the average of the control delta values for the lowest concentration tested to 6% of the control delta values for the highest concentration tested. For the isolates from patients C, D, E, and G, average delta values for nalidixic acid ranged from 92% of the control for the lowest concentration tested to 23% of the control for the highest concentration tested.

Growth on selective media. On LJ, growth was apparent for all eight patient isolates by day 14, and for four of the eight isolates tested, the growth was first observed on LJ (Table 3). For the remaining four isolates, growth was observed first on LJ and at least one other medium. Growth was observed latest on Mycobactosel LJ for six of the eight isolates. One of the remaining two isolates was detected latest on both Mycobactosel LJ and LJ Gruft; the other was detected latest on both Mycobactosel LJ and Mycobactosel.

All media which had no visible growth until day 36 had significantly less growth than the LJ control (Table 3).

DISCUSSION

The BACTEC radiometric system has been shown to provide a significant reduction in detection time for mycobacteria

TABLE 3. Day of detection for solid media inoculated with suspensions of *M. kansasii*

Patient isolate	Day of detection on:			
	LJ	Mycobactosel	Gruft	Mycobactosel LJ
A	11	27	36[a]	36[a]
B	14	18	27	36[a]
C	11	11	14	27[a]
D	11	11	14	18
E	11	14	14	27
F	11	36[a]	14	36[a]
G	11	11	11	27
H	14	14	14	18

[a] Diminished growth relative to growth on LJ.

compared with conventional culturing techniques (2, 3). Our experience with the relatively slow growth of two patient *M. kansasii* isolates in 12B with PANTA added prompted this investigation of a possible inhibitory factor for *M. kansasii* in PANTA or in 12B itself.

Our analysis of the factors affecting the growth of these *M. kansasii* isolates suggested that some component of PANTA was exerting an inhibitory effect. We expanded our study to include six other patient isolates and an American Type Culture Collection isolate to determine the extent of this inhibition (Table 1).

Further studies with various concentrations of the individual antibiotics contained in PANTA suggested that it was the nalidixic acid which was responsible for the inhibitory action of PANTA on the growth of *M. kansasii*. In an analysis of the susceptibility testing results from the two isolates which had shown some inhibition in response to PANTA, we compared the delta values of the drug-containing bottles with the delta values of the drug-free controls which received the same inoculum concentration (Table 2). For each concentration of polymyxin B, amphotericin B, trimethoprim, and azlocillin tested, the average delta value of the drug-containing bottle was >75% of the average delta value of the control bottles. This suggests that these antibiotics did not significantly affect the growth of the *M. kansasii* isolates. For nalidixic acid, however, the average delta GI of the lowest concentration tested (10 μg/ml) was only 27% of that of the drug-free control. As the concentration of nalidixic acid increased, the average delta GI decreased to an average of 6% of that of the control for the highest concentration tested (40 μg/ml), suggesting increasing inhibition of the organism.

When the effect of nalidixic acid on the remaining patient isolates was tested, some inhibitory effects were also noted (Table 2). While the lowest concentration tested (10 μg/ml) gave an average delta GI which was 92% of that of the control, the highest concentration tested (40 μg/ml) gave an average delta value which was only 23% of that of the control. At the concentration of nalidixic acid present in 12B bottles with PANTA added (20 μg/ml), the average delta value for these isolates was only 61% of that of the controls. Although time to detection of these isolates was not significantly affected by the nalidixic acid present in the media, there was obviously some reduction in the rate of growth compared with that of the drug-free control.

While it appears unlikely that any *M. kansasii* isolate will be completely inhibited by the recommended concentration of PANTA in 12B bottles, it is apparent that some isolates are sufficiently sensitive to nalidixic acid to result in a delay of detection of at least several days. The degree to which isolates are affected by PANTA may be affected by factors intrinsic to the organisms being tested. In this study, detection of the isolates from patients A and B was most delayed by the addition of PANTA, while the detection of isolates from patients C and E was affected to a lesser extent (Table 1). The isolates from patients D, G, and H appeared to be unaffected by PANTA; they were detected earlier by the BACTEC system than on solid media. The degree of growth inhibition may also be affected by the concentration of organism inoculated into the 12B bottle.

We attempted to determine the inhibitory effect of the antibiotics present in certain mycobacteriologic selective media on the growth of all eight patient isolates of *M. kansasii*. Growth from a diluted, standardized suspension inoculated onto Mycobactosel, Mycobactosel LJ, and LJ Gruft was compared with growth on LJ. While all isolates showed good growth on LJ by the 14th day of incubation, all showed delayed growth on at least one of the antibiotic-containing media. Growth was observed last on Mycobactosel LJ from six of the eight isolates tested, and seven of the eight isolates first showed growth on this medium at least a full week after the LJ became positive (Table 3). In addition, there was significantly less growth on the media becoming positive at day 36 than was present on the LJ control slant inoculated at the same time. While we did not determine the susceptibility of *M. kansasii* to the other antimicrobial agents in these media, it appears that at least one of the antimicrobial agents present in these media can exert an inhibitory effect on *M. kansasii*. On the most inhibitory medium, Mycobactosel LJ, the combined high concentrations of nalidixic acid and cycloheximide may have contributed to the substantial inhibition of growth.

It is interesting that the isolate from patient A, which was determined to be susceptible to nalidixic acid at 40 μg/ml, was also the isolate most significantly inhibited on antibiotic-containing media. LJ Gruft and Mycobactosel LJ, both of which contain 35 μg of nalidixic acid per ml, appeared to be the most inhibitory to this isolate, resulting in both delayed and diminished growth.

To ensure the most rapid detection of *M. kansasii* from clinical specimens, it is important to include a nonselective medium in the initial culture setup and to carefully inspect the nonselective solid media early in the incubation period. Selective media should be carefully examined far into the incubation period to detect delayed and/or diminished growth. In addition, the recommended concentration of PANTA added to 12B bottles at the time of processing should be carefully controlled, because too much of this additive may further delay the detection of *M. kansasii*. Users should also be aware that increasing the concentration of PANTA in 12B bottles as recommended by the manufacturer (4) to control the overgrowth of contaminants from clinical specimens may compromise the recovery of *M. kansasii* from those specimens.

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